

AWARD NUMBER: W81XWH-13-1-0468

TITLE: The Role of Protein Radicals in Chronic Neuroimmune Dysfunction and Neuropathology in Response to a Multiple-Hit Model of Gulf War Exposure

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REPORT DATE: October 2014

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE CW 2014		2. REPORT TYPE Annual		3. DATES COVERED 30 Sep 2013 - 29 Sep 2014	
4. TITLE AND SUBTITLE The Role of Protein Radicals in Chronic Neuroimmune Dysfunction and Neuropathology in Response to a Multiple-Hit Model of Gulf War Exposure				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-13-1-0468	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Michelle L. Block E-Mail: mlblock@iupui.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Virginia Commonwealth University 800 East Leigh Street, Suite 3200 P.O. Box 980568 Richmond, VA 23219				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Chronic peripheral inflammation and neuroinflammation have been linked to Gulf War Illness (GWI), but the underlying mechanisms are unknown. A Multiple-Hit Hypothesis is supported, where the synergistic interaction of several potential neurotoxins and triggers of inflammation, such as persistent peripheral inflammation and the organophosphate pesticide chlorpyrifos (CPF) may interact to culminate in diverse symptoms. As a consequence of this biological and chemical complexity, early/accurate diagnosis and effective treatments have proven elusive. Our research over the last year has addressed these issues in GWI with a "Multiple-Hit" mouse model of GWI-like exposures and focused on the key molecular target NF-κB p50 in CNS effects. Experiments employing a mouse model of GWI for short term and long term analysis of the brain after GWI-like exposures have been completed for AIM1 along with the collection of circulating white blood cells for AIM3. While sample analysis is ongoing, preliminary results suggest that the hippocampus in NF-κB p50-/- mice is more vulnerable to chronic neuroinflammation at one week after pro-inflammatory insult. We also report that CPF impairs NF-κB p50 function in microglia, suggesting that CPF may predispose the hippocampus to chronic neuroinflammation. We will directly test this hypothesis as the collected samples are further analyzed.					
15. SUBJECT TERMS Gulf War Illness Mouse Model, Chlorpyrifos, LPS, NF-KB p50, microglia, chronic neuroinflammation, serum markers, neuropathology					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	10	19b. TELEPHONE NUMBER (include area code)

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1. Introduction

Chronic peripheral inflammation and neuroinflammation have been linked to Gulf War Illness (GWI), but at present there is little information regarding the underlying mechanisms of this multifaceted illness. Current opinion supports a Multiple-Hit Hypothesis, where the synergistic interaction of several potential neurotoxicants and triggers of inflammation, such as persistent infections, and the organophosphate pesticide chlorpyrifos (CPF) may interact to culminate in diverse symptoms. As a consequence of this biological and chemical complexity, early/accurate diagnosis and effective treatments have proven elusive. This research proposal begins to address these issues in GWI using an *in vivo* approach with a “Multiple-Hit” model of Gulf War exposures that focuses on the key molecular target, NF-κB p50 in the brain. NF-κB is a redox-sensitive, prototypical pro-inflammatory transcription factor family associated with inflammation-mediated CNS pathology. Our recent work has shown that oxidized NF-κB p50 is linked to loss of function and a CNS-specific vulnerability to chronic inflammation. Interestingly, we have also shown that the GW exposure CPF oxidizes NF-κB p50 in the brain’s innate immune cell, microglia. The first year of this research was dedicated to AIM1 to discern the role of NF-κB p50 in the early and late CNS effects present in our multiple hit mouse model of GWI.

2. Keywords

Gulf War Illness mouse model; Chlorpyrifos; LPS; NF-κB p50; microglia; chronic neuroinflammation; serum markers; neuropathology

3. Accomplishments

I. Relevant Major Project Goals

Objectives per the Statement of Work

Research will be restricted to the following objectives:

- 1) assessing mechanisms responsible for the synergistic interactions of the Gulf War-like Exposures lipopolysaccharide (LPS) and chlorpyrifos (CPF) (AIM1, Task1);
- 2) showing potential therapeutic utility in inhibiting NOX2 to attenuate chronic neuroinflammation and neuron damage in response to the GW exposures LPS & CPF (AIM2, Tasks 2 &3);
- 3) demonstrating the feasibility of the NFκB p50 radical as a peripheral marker for ongoing neuroinflammation and neuropathology in response to the Gulf War exposures LPS and CPF (AIM3, Task 4).

Percentage Completion of Designated Tasks in Year 1

Task 1a. The mouse experimental work for AIM1 was completed. (100% Completion)

Task 1b. Processing and analysis of the samples for AIM1 is in progress. (Estimated 15% Completion) Sample processing was delayed due delay of experimental completion from VCU facility issues and relocation of the PI’s laboratory.

Task 4a. Samples collected for AIM3 (100% Completion of Year 1 Task, Expected 33% of total task effort)

II. Accomplishments Under the Goals

i. Major Activities

Animals Treated: Male mice missing the NF- κ B p50 protein and the control strain (C57) were treated with our Multiple-Hit Model of Gulf-War Exposures treatment paradigm: All mice were treated with either LPS (1mg/kg, i.p.) or Saline (i.p.) at 2 months of age. One week after the injection, after the peripheral (but not the central) immune response has been shown to resolve, mice will receive 5 daily injections of either CPF (5mg/kg, s.c.) or peanut oil vehicle (s.c.). The resulting treatment groups were: Saline, LPS, CPF, peanut oil, and CPF + LPS.

Necropsy & Brain Sectioning: Immediate (early - 3 hours after last CPF injection) and delayed (chronic – 3 months post CPF treatment) animals were euthanized and half of the brain was fixed and sectioned for immunohistochemistry (IHC); half of the brain was sectioned into the 5 brain regions (olfactory bulb, midbrain, frontal cortex, temporal cortex, and cerebellum) and snap frozen for mRNA and protein analysis.

Preliminary Analysis of Early Time Point Data: Measures of microglial activation and neuroinflammation at the early 3 h time point was assessed in some samples to confirm success was probable with the experimental design and dose. The full measures of neuroinflammation, oxidative stress, and hippocampal neuropathology will be assessed at both age points to discern the role of NF κ B p50 in pathology.

ii. Specific Objectives

Task I: Multiple-Hit Model of Gulf-War Exposures Tested in NF κ B p50^{+/+} and NF κ B p50^{-/-} Mice (AIM1 - Timeframe: Year 1) In this task, male mice missing the NF κ B p50 protein and the control strain (C57) were treated with our Multiple-Hit Model of Gulf-War Exposures treatment paradigm.

This task was expected to take the entire first year to complete.

1a. The mouse experiment was expected to take approximately 5 months to complete after receipt of the mice.

1b. Processing and analysis of the samples was predicted to take 7 months.

Task IV: Processing Peripheral Blood Cells & Measuring the NF κ B p50 radical (AIM3; Timeframe: Years 1- 3)

This task is expected to take all 3 years to complete.

4a. Fresh blood samples collected in Tasks I-III will be processed with DMSO immediately at the time of the experiment.

4b. Protein will be frozen back for analysis, but samples for Task I will be analyzed at the end of Year 1, TaskII at the end of Year 2, and Task III at the end of Year 3.

Percentage Objective Completion:

Task 1a. The mouse experiment was completed. (100% Completion). It took 9 months to complete with incomplete numbers of animals in each group due to unexpected animal and general facility issues at VCU. We should have sufficient N to analyze the endpoints for most measures proposed.

Task 1b. Processing and analysis of the samples is in progress. (Estimated 15% Completion) This was unexpectedly delayed due to issues with the animal facilities resulting in experimental loss and significantly impaired ability to generate mice/run experiments to collect samples to analyze. At present, assessment of samples is also delayed due to relocation of the PI's laboratory to the Indiana University School of Medicine in Indianapolis, Indiana. Once funds are released from VCU, we should be able to resume analysis at the same time we are completing task 2.

Task 4a. Samples collected (100% Completion of Year 1, Expected 33% of total task effort)

Task 4b. N/A To be analyzed in year 3.

iii. Significant Results/Key Outcomes

NFκB p50^{-/-} mice are sensitive to chronic neuroinflammation in the hippocampus:

We confirmed with preliminary analysis that at 1 week post LPS injection of the low dose (1mg/kg, IP) only the NFκB p50^{-/-} mice showed chronic neuroinflammation in the hippocampus, confirming vulnerability to lower grade peripheral inflammation.

The samples for AIM 4 are waiting for later processing as expected and the majority of samples for AIM1 are still being processed.

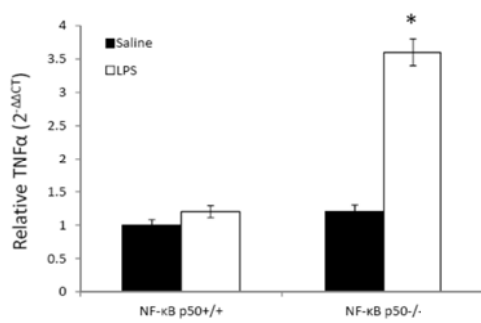


Figure 1. NF-κB p50^{-/-} are sensitive to LPS-induced chronic neuroinflammation in the hippocampus. Mice were injected with saline or LPS (low dose, 1mg/kg IP) and TNFα was assessed in the hippocampus a week later by quantitative rtPCR. An * denotes significance from control (P<0.05)

- iv. Other Achievements
N/A

III. Opportunities for Training and Professional Development

Graduate Student Training: The graduate student Savannah Brookins had individual training with the PI and staff on animal injections, data analysis, and research presentation skills (including a poster at the SOT meeting presented about the preliminary data).

GW-Specific Meeting: Block ML. (April, 2014). Invited Speaker for the Research Advisory Committee on Gulf War Veterans' Illnesses Meeting, Washington, DC

GW Grant Reviewer: Block ML (December 2013) Reviewer, DoD Congressionally Directed Medical Research Programs, Gulf War Illness Research Program, Investigator-Initiated Research Award

IV. Dissemination of Results

The preliminary data was paired with in vitro studies to present this work at the annual Society of Toxicology Meeting and the VA.

- A. Block ML. (April, 2014). Invited Speaker for the Research Advisory Committee on Gulf War Veterans' Illnesses Meeting, Washington, DC
- B. Brookins S, Taetzsch T, Levesque S, McGraw C, & Block ML. (March 2014). Chlorpyrifos Oxon Primes Microglia: Enhanced LPS-Induced TNF α Production. Poster presentation for the SOT meeting, Phoenix, AZ
- C. Brookins S, Taetzsch T, Levesque S, McGraw C, & Block ML. (December 2013). Chlorpyrifos Oxon Primes Microglia: Enhanced LPS-Induced TNF α Production. Poster presentation for the Central Virginia Society for Neuroscience meeting, Richmond, VA

V. Plan for Next Year to Accomplish Goals

The PI's laboratory and the collected samples have moved to IUSM specifically because of the high quality of the animal facilities at IUSM and the ability to support this research. Once the award transfers to IUSM, we will process the previously collected samples while simultaneously moving forward here with AIM2 and the second component to AIM4 as originally planned. We believe that the increased productivity due to enhanced facility support with the lab relocation will allow us to close the gap and gain the opportunity to move forward as expected and outlined in the SOW.

4. Impact

Impact on Science (neuroscience & other disciplines): The studies in AIM1 are beginning to provide much needed insight into the underlying mechanisms driving why the CNS effects of chemical and pro-inflammatory exposures may persist long after the instigating stimulus, such as what occurs with GWI.

Impact on Technology Transfer

N/A Nothing to Report

Impact on Society – Specifically GW Veterans: In addition, to revealing potential therapeutic targets in AIM1, this work took the first steps of AIM3 that will explore the potential utility of the NFkB p50 protein radical as an early peripheral biomarker of chronic, delayed, and deleterious CNS effects, all of which we believe will provide foundation for future translational GWI studies.

5. Changes/Problems

I. Changes in Approach

Nothing to report

II. Problems, Delays, and Action Plans to Solve Them

A. Problems:

- i. **Minor adjustments to vehicle and dose:** There were unforeseen minor adjustments necessary due to the unique characteristics of a vehicle and the strain of mouse being tested. We discovered that peanut oil must be used as a vehicle instead of DMSO (DMSO and CPF displayed an unexpected confounding interaction effect on neuroinflammation) and that the LPS dose must be lowered to 1mg/kg IP due to extreme sensitivity of the NFkB p50^{-/-} mice and unexpected mortality. **Solution:** These very minor adjustments caused no significant delay and allowed the proposed approach, objectives, and scope of the study to continue as planned.
- ii. **Animal facility issues:** Facility failures led to unexpected termination of ongoing studies and obligated the laboratory to start over on multiple occasions. The setbacks were: 1) Repeated/chronic unannounced renovations in Sanger Hall disturbing chronic studies resulting in both the cessation/loss of ongoing work and stalled initiation of animal studies; 2) There was a November 2013 water main break resulting in loss of both general power and emergency power for 24 hours, where the consequence was the inability work in the building due to chemical hazards from basement flooding and electrical work for 1 month. This terminated ongoing animal research studies and halted bench research

for a month. 3) There were several unsuccessful attempts to resolve reoccurring facility issues. **Solution:** The amount of renovations, training, resource expenditures, and time that would be necessary for VCU to support this research was determined to be prohibitive. The PI's laboratory has moved from VCU to the Indiana University Medical School in Indianapolis, Indiana and the Stark Neuroscience Research Institute.

III. Changes Significantly Impacting Expenditures

- a. **Increased Animal Research Spending:** The reoccurring unexpected loss of animal research, the consequent cost to attempt to replace, and the cost of the failed attempt to relocate the PIs animal research program to the MMRB building at VCU significantly elevated the animal research costs.
- b. **Change in Employment:** The PI and staff left VCU on July 31, 2014. Unexpected additional charges for payout of the PI and staff's paid leave were applied to the award funds.

IV. Significant Changes in Use or Care of Human Subjects

Nothing to report

V. Significant Changes in Use or Care of Vertebrate Animals

Nothing to Report

6. Products

N/A Nothing to Report

7. Participants & Other Collaborating Organizations

I. Individuals Working on the Project

Name	Michelle L. Block	Shannon Levesque	Rafy Luqa	Savannah Brookins
Project Role	PI	Technician	Animal Tech	Grad Student
Effort (months)	3	12	12	0
Contribution	Manage/oversee project and all personnel	Managed laboratory, completed bench work, moved colony/trouble shot facility issues, trained animal tech/grad student, and completed some preliminary studies	Performed animal research experiments, supervised the colony maintenance	Assisted /performed/trained in animal research. Analyzed data, presented research findings
Other support	NIEHS/NIH (6 months effort) VCU University (2 months effort)	N/A	N/A	VCU University (12 Months effort)

II. Change in Active Support

Nothing to report.

III. Partner Organizations

Nothing to report.

8. Special Reporting Requirements

Nothing to report.

9. Appendices

Nothing to report.